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Pyridine Nucleotide-Dependent Ferricyanide Reduction Associated with Isolated Plasma Membranes of Maize (*Zea mays* L.) Roots

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Summary

Plasma membranes (PM) from maize roots (*Zea mays* L.) were isolated by aqueous two-phase partitioning. The isolated membrane fraction showed a 4.6-fold enrichment in specific activity of the PM marker enzyme vanadate-sensitive, Mg^{2+}-ATPase over a microsomal pellet collected at 50,000 × g. Activities of marker enzymes for mitochondria, endoplasmic reticulum, tonoplast, and Golgi apparatus were low or not detectable in the PM fraction. Quantitative morphometric analysis using the PM-specific silicotungstic acid stain showed the fraction to be > 92% PM vesicles. Using detergent stimulation of ATPase activity as a measure of structurally linked latency, greater than 90% of the PM vesicles were oriented with the cytoplasmic surface inside.

An electron transport activity was investigated in the PM fraction. The rate of NADH oxidation in the absence of an artificial electron acceptor was < 167 pkat · mg protein^{-1}; however, NADH catalysed the reduction of a variety of artificial electron acceptors including ferricyanide (2.6 nkat · mg protein^{-1}), cytochrome *c* (0.8 nkat · mg protein^{-1}), a tetrazolium derivative (0.6 nkat · mg protein^{-1}) and dichlorophenol indophenol (0.4 nkat · mg protein^{-1}). While the NADH-dependent ferricyanide and dichlorophenol indophenol reductases were stimulated ⩾ 6-fold by 0.025% (v/v) Triton X-100, the cytochrome *c* and INT reductases were not greatly stimulated. Washing membranes with high salt significantly decreased the NADH-dependent, and eliminated the NADPH-dependent, ferricyanide reductase activity measured in the absence of detergent. These results suggest that NADH was oxidized on the extracytoplasmic surface of the membrane; however, a significant portion of this activity was extrinsic and may have originated from cytoplasmic contamination during isolation. The greater portion of the PM-associated NAD(P)H oxidation and/or ferricyanide reduction was catalyzed on sites not exposed to the outer surface of the membrane.

Keywords: Aqueous two-phase partitioning; Ferricyanide reductase; NAD(P)H oxidoreductase; Plasma membrane; Redox system; Root (membranes).

1. Introduction

NAD(P)H oxidoreductases have been identified on nearly all plant membranes (MØLLER and LIN 1986). The most extensively investigated NAD(P)H oxidoreductases are those associated with the inner and outer mitochondrial membranes and with the endoplasmic reticulum. NAD(P)H oxidases associated with both the inner and outer surfaces of the inner mitochondrial membrane are linked to cytochrome a/a_3 oxygenase and are coupled to ATP synthesis, while the function of the NADH oxidase associated with cytochrome *b* on the outer membrane is not known (MØLLER and LIN 1986). The NAD(P)H-cytochrome *c* reductase on the endoplasmic reticulum has been used widely as a marker enzyme in organelle isolation

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Abbreviations: BTP = 1,3-bis[tris(hydroxymethyl)-methylamino]-propane; CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate dihydrate; cyt *c* = cytochrome *c*; DCIP = 2,6-dichlorophenol indolphenol; INT = 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; kat = mole · s^{-1}; Mes = 2-(N-morpholino)ethanesulfonic acid; MF = microsomal fraction; PM = plasma membrane; STA = silicotungstic acid; Tris = 2-amino-2 -(hydroxymethyl)-1,3-propanediol.

(QUAIL 1979). This activity is analogous to the cytochrome b_5 or P-450 systems of the mammalian endoplasmic reticulum, and functions in hydroxylation and desaturation reactions (MØLLER and LIN 1986). NAD(P)H dehydrogenase activity has been reported in vacuoles of *Hevea* (MOREAU *et al.* 1975), the Golgi apparatus of rat liver (MORRÉ *et al.* 1978; BARR *et al.* 1984), but thus far not in plant Golgi, and glyoxysomal membranes of *Ricinus* where transfer of reducing equivalents to the cytoplasm *via* a NADH oxidase system is proposed as part of the glyoxylate cycle (LUSTER and DONALDSON 1985).

Of particular interest to this study, are the reports of NAD(P)H dehydrogenase activity on plant PM (reviewed by CRANE *et al.* 1985). The presence of a PM-associated redox system in plants has been demonstrated in intact roots (RUBINSTEIN *et al.* 1984), whole cells (BARR *et al.* 1985 a, MISRA *et al.* 1984), protoplast (LIN 1982 and 1984, THOM and MARETZKI 1985) and isolated membranes (DELUCA *et al.* 1984, RUBINSTEIN *et al.* 1984). Furthermore, BARR *et al.* (1985 b) demonstrated a PM redox activity in a well characterized, highly enriched PM fraction isolated from soybean hypocotyls. Taken together, the results present evidence for a redox pathway on PM, although the biochemical characteristics and the physiological function of the PM redox system remain obscure.

In this study, we report findings of an analysis of the NAD(P)H oxidoreductase activity in a PM fraction prepared from maize roots using the aqueous two-phase partitioning method and suggest that the major portion of the NAD(P)H-dependent reductase activity is not accessible from the extracytoplasmic surface of the PM.

2. Materials and Methods

Plant material. Seeds of maize (*Zea mays* L. cv Golden Cross Bantam) were surface sterilized in a 10% (v/v) solution of commercial bleach, rinsed with tap water and soaked in running tap water for *circa* 18 hours. Seeds were then drained, spread onto stainless steel wire-mesh racks, covered with a single layer of Miracloth (Behring Diagnostics, La Jolla, CA) and grown in darkness at 27 °C for 3 days. Primary roots were harvested and stored on ice until needed. Storage time did not exceed 1 hour.

Microsomal membrane isolation. Root material was chopped using hand-held, single-edged razor blades in homogenization buffer (15 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, 3 mM dithiothreitol, 0.5 M sucrose and 0.6% insoluble polyvinylpolypyrrolidone) at a ratio of 1 : 5 (w/v). The chopped root material was vacuum infiltrated twice at 650 mm of Hg for 15 s followed by Polytron homogenization (Brinkmann Instruments, Westbury, NY) for 30 s at rheostat setting 6. The homogenate was filtered through nylon cloth (pore size 210 µm) and centrifuged at 10,000 g_{max} for 10 minutes (Sorvall SS-34 rotor, Du Pont Co., Wilmington, DE). This supernatant was then centrifuged at 50,000 g_{max} for 30 minutes (SS-34 rotor). The resulting pellets were resuspended in approximately 6 ml of 5 mM K-phosphate buffer at pH 7.8 containing 0.25 M sucrose and were referred to as the microsomal fraction (MF).

Plasma membrane isolation. PM was isolated using the aqueous two-phase partitioning method described by KJELLBOM and LARSSON (1984) with slight modifications. This technique has been reviewed by ALBERTSON *et al.* (1981) and LARSSON (1983). For the phase partitioning, two 18 g phase systems were constructed containing 6.2% dextran T 500 (Pharmacia AB, Uppsala, Sweden) 6.2% polyethylene glycol with an average molecular mass of 3350 Da (Sigma Chemical Co., St. Louis, MO), 0.048% phosphate buffer titrated to pH 7.8 with KOH, 0.037% KCl and 8.56% sucrose all calculated by weight. Five g of membranes suspended in the phosphate buffered sucrose solution were added to a third phase system to give an 18 g system of the composition described above. All systems were equilibrated to 2 °C and partitioned by vigorous shaking for 20 s. The phase were separated by centrifugation at 1,000 g_{max} for 5 minutes at 2 °C (Sorvall HB-4 rotor, Du Pont, Wilmington, DE). All upper phases were removed and stored separately at 2 °C. For the second partition, the upper phase containing membrane material was applied to a fresh lower phase, equilibrated, partitioned and separated as above. The upper phase from the second partition was again applied to a fresh lower phase and treated as above. The resulting upper phase was referred to as U_3 using the terminology of KJELLBOM and LARSSON (1984). The partitioning procedure is illustrated in Fig. 1. In a parallel series of partitions, the lower phase obtained from the first partitioning step was re-extracted with fresh upper phase, equilibrated, partitioned and separated as above. The twice-extracted lower phase was recovered as fraction L_1 (Fig. 1), and the upper phase from this extraction was applied to the lower phase obtained from the second partition of U_3 and repartitioned. The upper phase from this partition was applied to the lower phase of the third partition of U_3 and partitioned as described. The resulting upper phase fraction was referred to as U'_3 (Fig. 1). The fractions MF, L_1, U_3 and U'_3 were diluted with buffer A (5 mM Tris-Mes, pH 7.0, 0.1 mM dithiothreitol and 0.25 M sucrose) for marker enzyme analysis or buffer B (5 mM Tris-Mes, pH 7.0, 0.2 mM $CaCl_2$, 1 mM KCl and 0.25 M sorbitol) for NADH dehydrogenase assays. Fractions were pelleted at 113,000 g_{max} for 30 minutes (SW 28 rotor, Beckman Instruments, Palo Alto, CA), and the pellets were resuspended in buffer A or B as needed. For NAD(P)H oxidoreductase analysis, membranes from fractions U_3 and U'_3 were combined and frozen at −80 °C until needed. Activity was stable for several months at this temperature. Membranes for marker enzyme analyses were used immediately after isolation without prior freezing.

Enzyme analyses. Marker enzymes for endoplasmic reticulum, Golgi apparatus and mitochondria were, respectively, antimycin A-insensitive, NADH-cytochrome c reductase (HODGES and LEONARD 1982), Triton X-100-stimulated IDPase (BOWLES and KAUS 1976) and succinate—INT—reductase (PENNINGTON 1961) performed by the modifications of BUCKHOUT *et al.* (1982). Vanadate-inhibited, Mg^{2+}-ATPase activity, a marker enzyme for PM, was assayed in 50 mM Mes-BTP, pH 6.0, buffer containing 3 mM $MgSO_4$, 1 mM NaN_3, 0.1 mM NH_4-molybdate and 0.25 M sucrose in the presence or absence of 0.025% (v/v) Triton X-100 and/or 100 µM Na_3VO_4. Nitrate-inhibited Mg^{2+}-ATPase, a marker enzyme for tonoplast membranes, was assayed at pH 7.0 in 50 mM Mes-BTP in the presence or absence of 50 mM nitrate with or without Triton X-100, and containing Mg^{2+}, sucrose, molybdate and azide as described. The assays were conducted in a volume of 100 µl containing 30 µl membrane fraction, initiated by the addition of ATP to 3 mM and run

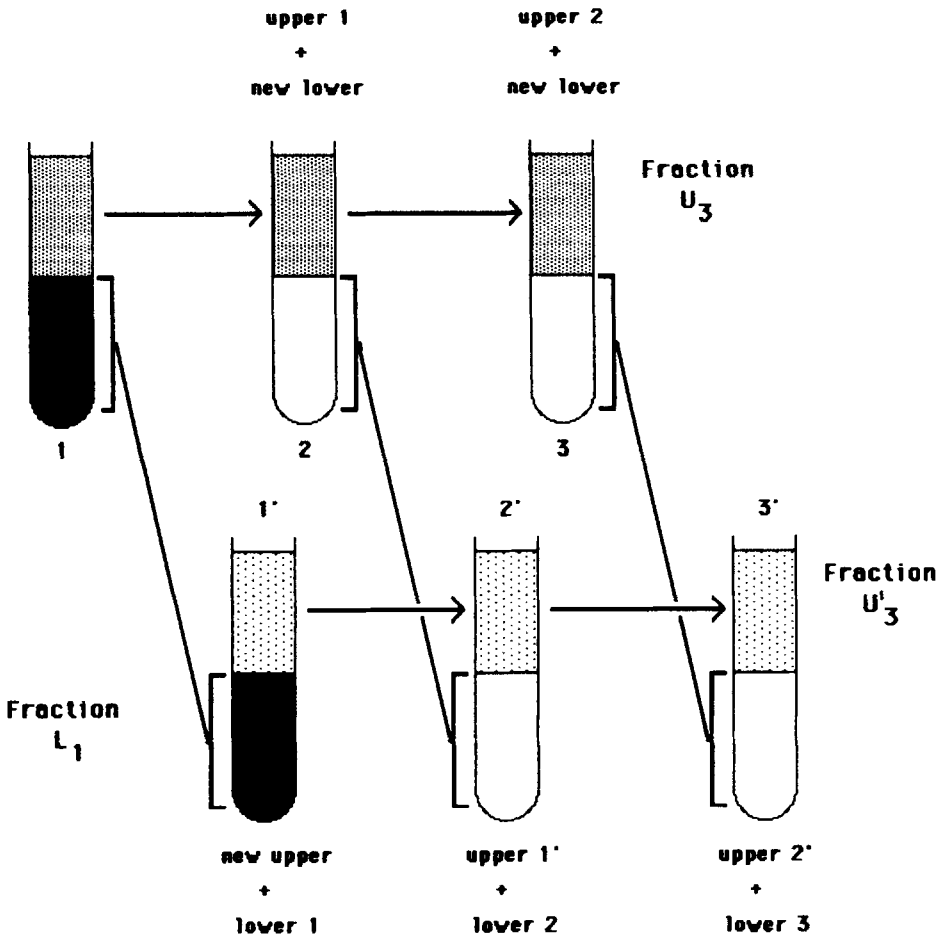

Fig. 1. Schematic diagram of the aqueous two-phase partitioning method used for isolation of PM from maize roots. A detailed discription of the procedure is provided in the Materials and Methods. The PM fraction is the combined fraction U_3 and U'_3

for 30 minutes at 30 °C. The reaction was stopped with the addition of 400 µl of ice-old 10% (w/v) trichloroacetic acid. Free phosphate was determined by the method of SCHERER and MORRÉ (1978). Protein was determined by the method of BRADFORD (1976).

NAD(P)H dehydrogenase was determined spectrophotometrically following the reduction of the artificial electron acceptors ferricyanide at 420 nm ($e = 1 \times 10^3 mol \cdot cm \cdot l^{-1}$), DCIP at 600 nm ($e = 2 \times 10^4 mol \cdot cm \cdot l^{-1}$), INT at 500 nm ($e = 1.15 \times 10^4 mol \cdot cm \cdot l^{-1}$) or cyt *c* at 550 nm ($e = 2.94 \times 10^4 mol \cdot cm \cdot l^{-1}$). Unless otherwise indicated, the assay for ferricyanide reductase was conducted with 5–15 µg membrane protein in buffer B containing 0.3 mM ferricyanide and 0.16 mM NAD(P)H plus or minus 0.025% (v/v) Triton W-100. The reaction was initiated by the addition of NAD(P)H.

KCl washing of PM. KCl extraction was conducted by resuspending PM in buffer B containing 0.5 M KCl and incubating on ice for 20 minutes, followed by centrifugation for 30 minutes at 110,000 g_{max} (SW 50.1 rotor, Beckman Instruments, Palo Alto, CA). The resulting pellet was resuspended in 200 µl of buffer B and the NAD(P)H-dependent, ferricyanide reductase activity in the pellets and supernatants was analyzed.

Electron microscopy. Isolated membrane pellets were resuspended in 50 mM cacodylate buffer, pH 7.2, containing 0.25 M sucrose and 2.5% (v/v) glutaraldehyde and fixed 1 hour at room temperature. The membrane suspension was pelleted by centrifugation for 30 minutes at 110,000 g_{max} (SW 50.1 rotor). These pellets were broken into small pieces (0.5 mm^2), washed in cacodylate buffer and postfixed in cacodylate-buffered 1% (w/v) osmium tetroxide for 1 hour at room temperature. Postfixed membranes were washed in water, dehydrated in acetone and embedded in Epon 812 resin (Poly/bed, Polysciences, Inc., Warrington, PA). Silver-gold sections were stained by the method of REYNOLDS (1963). Silicotungstic acid staining was performed by the method of ROLAND (1978). This stain results in a similar staining specificity for PM as does phosphotungstic acid (ROLAND *et al.* 1972), but in our opinion gives somewhat sharper contrast. Morphometric analysis was performed by the method of WIEBEL *et al.* (1966).

3. Results

Isolation and characterization of plasma membranes. PM were isolated by aqueous two-phase partitioning, as described in the Materials and Methods and illustrated in Fig. 1. We present the following evidence confirming the purity of this fraction. First, enzymatic analyses show that < 0.15% of the total succinate-

Table 1. *Analysis of marker enzymes in membrane fractions isolated by the aqueous two-phase partitioning procedure. Numbers in parentheses are specific activities. Recovery of activity was < 100% in most cases because all upper and lower phases from the multiple partitioning steps were not analyzed*

Fraction	Protein (mg)	Triton X-100-stimulated, IDPase[a]	NADH-cytochrome C reductase[b]	Succinate INT-reductase[a]
MF	56.22	63,795.0 (1,134.67)	207.0 (3.67)	7,448.33 (132.5)
% recovered	100.00	100.00	100.00	100.00
L_1	40.33	26,967.5 (668.67)	125.83 (312.17)	7,741.67 (192.0)
% recovered	71.74	42.27	60.80	103.94
U_3	2.39	114.17 (47.50)	1.32 (0.55)	9.0 (3.33)
% recovered	4.25	0.18	0.63	0.11
U'_3	1.48	51.17 (34.67)	0.53 (0.37)	0.83 (0.5)
% recovered	2.62	0.08	0.26	0.01

[a] pkat (pkat · mg protein $^{-1}$); [b] nkat (nkat · mg protein^{-1}).

Table 2. *Analysis of ATPase marker enzymes for tonoplast in membrane fractions isolated by aqueous two-phase partitioning. ATPase activity was determined as the release of phosphate in the absence or presence of 0.025% Triton X-100 (%, v/v). Numbers in parentheses are specific activities*

Fraction	Mg^{2+}-ATPase (pH 7.0)[a]			
	Basal Triton X-100		Nitrate-inhibited, Triton X-100	
	—	+	—	+
MF	448.6 (8.0)	872.7 (15.5)	82.2 (1.46)	71.4 (1.3)
% recovered	100.00	100.00	100.00	100.00
L_1	313.4 (7.8)	484.8 (12.0)	47.1 (1.2)	68.8 (1.7)
% recovered	69.9	55.5	57.3	96.4
U_3	8.7 (3.6)	91.5 (38.2)	—0.8 (—0.3)	—0.8 (—0.3)
% recovered	1.9	10.5	—	—
U'_3	9.2 (2.0)	54.9 (37.3)	—0.5 (—0.4)	—6.1 (—4.2)
% recovered	2.0	6.3	—	—

[a] nkat (nkat · mg protein $^{-1}$).

INT-reductase (mitochondria), < 0.9% of the antimycin A-insensitive, NADH-cytochrome *c* reductase (endoplasmic reticulum) and < 0.3% of the Triton X-100-stimulated IDPase (Golgi apparatus) in the MF was recovered in the combined fractions U_3 and U'_3 (Table 1). Similarly, the specific activities of these enzymes were 10- to > 100-fold reduced in the U_3 and U'_3 fractions compared to the MF (Table 1). Recovery of activity was < 100% in most cases because all upper and lower phases from the multiple partitioning steps were not analyzed. Contamination by tonoplast of the PM fraction was estimated using the nitrate-inhibited Mg^{2+}-ATPase (Table 2). Nitrate inhibited the ATPase activity in MF and L_1 by 10 to 20% while stimulating the activity in the U_3 and U'_3 by approximately 10% as demonstrated by the negative values in Table 2. This stimulation was relatively small but consistently observed. More dramatic nitrate inhibitions were observed in membranes which pelleted from the 50,000 g_{max} supernatant (data not shown). The lack of nitrate-inhibited ATPase in U_3 and U'_3 demonstrates the absence of tonoplast membranes in these fractions.

Table 3. *Analysis of ATPase marker enzymes for PM in membrane fractions isolated by aqueous two-phase partitioning. ATPase activity was determined as the release of phosphate in the absence or presence of 0.025% Triton X-100 (%, v/v). Numbers in parentheses are specific activities*

Fraction	Mg^{2+}-ATPase (pH 6.0)[a]			
	Basal Triton X-100		Nitrate-inhibited, Triton X-100	
	—	+	—	+
MF	389.9 (6.9)	1,095.6 (19.5)	204.4 (3.6)	809.5 (14.4)
% recovered	100.00	100.00	100.00	100.00
L_1	319.2 (7.9)	502.6 (12.5)	202.1 (5.0)	345.0 (8.6)
% recovered	81.9	45.9	98.8	42.7
U_3	10.5 (4.4)	169.1 (70.7)	11.0 (4.6)	158.9 (66.4)
% recovered	2.7	15.4	5.4	19.6
U'_3	7.38 (5.0)	106.2 (72.0)	9.4 (3.6)	99.5 (67.5)
% recovered	1.9	9.7	4.6	12.3

[a] nkat (nkat · mg protein^{-1}).

Greater than 50% of the Mg^{2+}-ATPase activity in all fractions was vanadate sensitive, indicating that the majority of molybdate- and azide-insensitive ATPase was of PM origin. In the absence of Triton X-100, 99% of the total ATPase activity was located in L_1. In the presence of detergent, 32% of this activity was found in U_3 and U'_3 and 43% in L_1. The vanadate-inhibited ATPase activity was stimulated by detergent 14- and 10-fold in U_3 and U'_3 while only 4.0- and 1.7-fold in the MF and L_1 fractions, respectively (Table 3). If the detergent stimulation is a measure of structurally linked latency (LARSSEN *et al.* 1984) and the vanadate-sensitive ATPase activity is uniquely associated with PM, between 90 and 93% of the PM vesicles are oriented with the cytoplasmic surface inside. The specific vanadate-sensitive ATPase activity is 4.6-fold enriched in U_3 and U'_3 over that of MF. Taken together, these data indicate that the U_3 and U'_3 fractions are predominately of PM origin.

The second line of evidence demonstrating the PM origin of U_3 and U'_3 is based on quantitative morphometry. To insure unbiased sampling, fixed membrane to be embedded, embedded fractions to be sectioned and regions of the sections to be photographed were selected randomly. Representative micrographs of U_3 stained with lead citrate (Fig. 2 *A*) or silicotungstic acid (Fig. 2 *B*) are presented. Fractions U_3 and U'_3 were morphologically indistinguishable. A consistent feature of U_3 was the presence of multivesicular vesicles (Fig. 2 *A*). In most cases these multiple vesicles stained STA positive (Fig. 2 *B*, arrows), although multiple vesicles were observed which contained morphologically recognizable organelles such as mitochondria. Flattened vesicles were observed in U_3 and U'_3 which were reminiscent of single Golgi apparatus cisternae (Fig. 2 *A*, arrows, and *B*). Quantitative morphometric analysis for STA-staining vesicles demonstrated that U_3 and U'_3 were composed of 93.4 ± 2.5 and 92.2 ± 4.5% STA-staining vesicles, respectively, while MF and L_1 contained 16.8 ± 7.3 and 13.0 ± 3.5% STA-staining vesicles (Table 4). These data support the contention that U_3 and U'_3 are composed of PM-derived vesicles. Based on the 4.6-fold increase in specific activity of U_3 and U'_3 over MF, one would expect 21% of MF to be PM, a value which is somewhat greater than the composition measured by quantitative morphometry, but within the statistical error of calculation (Table 4).

NAD(P)H-dependent redox activity. Prior investigations (LIN 1982) have presented evidence to support the presence of a PM redox system on maize plasmalemma analogous to the system reported for animal cell PM. To better understand the biochemical structure of the maize root redox system and its physiological function, we have begun an analysis of the PM redox system using isolated PM vesicles. Oxidation of NAD(P)H or the reduction of artificial electron acceptors was investigated. Oxidation of NADH occurred at a rate of 33 to 167 pkat · mg protein^{-1} in the absence of an artificial electron acceptor. The rates of NADH-dependent

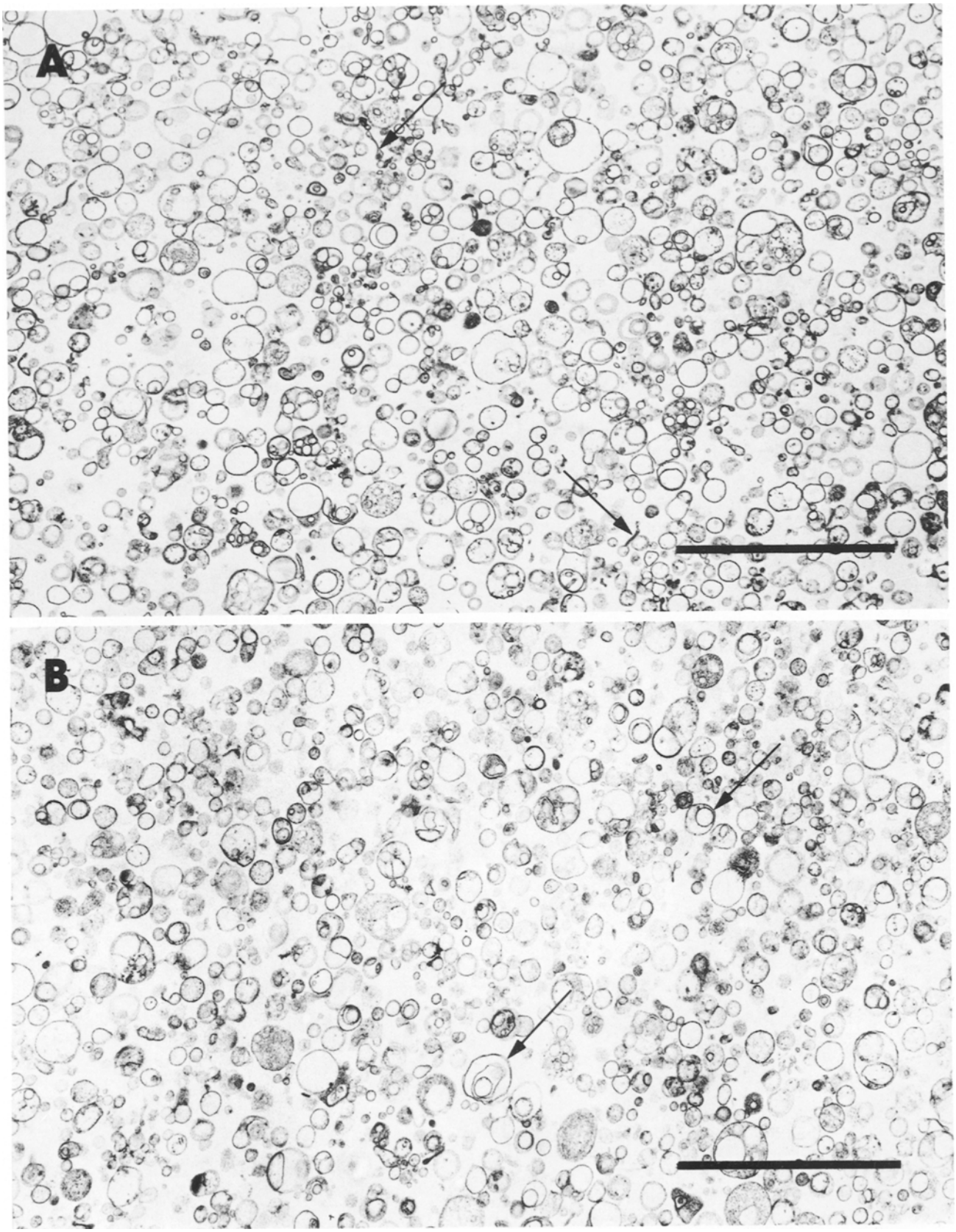

Fig. 2. Electron micrographs of membrane fraction U_3. Membrane fractions were isolated as described in Materials and Methods and illustrated in Fig. 1. U_3 membranes were fixed in glutaraldehyde and postfixed in osmium tetroxide. Silver-gold sections were stained with lead citrate (*A*) or STA (*B*) specific for PM. Fractions U_3 and U'_3 were morphologically indistinguishable. The primary magnification was 4,300× for both micrographs. Bars equal 3 μm

Table 4. *Analysis of silicotungstic acid staining in membrane fractions isolated by aqueous two-phase partitioning. Membrane fractions corresponding to those illustrated in Fig. 1 were prepared for electron microscopy as described in the Materials and Methods and analyzed by quantitative morphometry for PM content using the PM-specific, STA stain. The results are presented as percent STA positive vesicles ± SE and are an average of 2 independent scorings of 3, randomly selected micrographs for each fraction*

Membrane fraction	STA positive (%)
MF	16.8 ± 7.3
L_1	13.0 ± 7.3
U_3	93.4 ± 2.5
U'_3	92.2 ± 4.5

ferricyanide, cyt *c*, INT and DCIP reduction were 2.6, 0.8, 0.58 and 0.42 nkat · mg protein^{-1}, respectively (Fig. 3 *A*). Within experimental error and where NADH oxidation could be measured without interference, the rate of pyridine nucleotide oxidation was sufficient to account for acceptor reduction. Ferricyanide reduction in the presence or absence of detergent (see below) was insensitive to Mn^{2+}, superoxide dismutase and catalase, discounting the participation of superoxide radicals in the reduction of ferricyanide (Table 5).

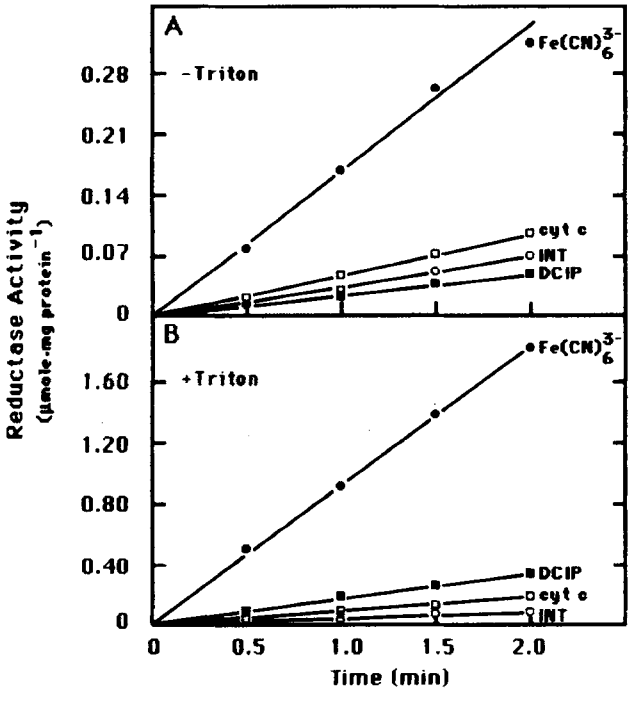

Fig. 3. Analysis of NADH-dependent reductase activity in PM. Isolated PM was analyzed for NADH-dependent ferricyanide (●), cyt c (□), INT (○) and DCIP (■) reductases as described in the Materials and Methods in the absence (*A*) and presence (*B*) of 0.025% Triton X-100 (v/v). Each data point is the average of 3 determinations

Table 5. *The effect of* Mn^{2+}, *catalase and superoxide dismutase on NADH-dependent, ferricyanide reductase activity. The ferricyanide reductase activity was assayed in PM as described in the Materials and Methods. Crystals of superoxide dismutase or catalase, or* Mn^{2+} *at a final concentration of 0.5 mM were added to the assay mixture and the ferricyanide reductase activity determined for 2 minutes. The results are an average of 3 determinations*

Treatment	Ferricyanide reductase activity[a]	
	Triton X-100	
	—	+
Control	2.80	21.47
Mn^{2+}	2.60	26.88
Superoxide dismutase	2.60	16.83
Catalase	3.23	19.67

[a] nkat · mg protein^{-1}.

Detergent-stimulated, NAD(P)H-ferricyanide reductase. The addition of Triton X-100 to a concentration of 0.025% (v/v) stimulated NADH-dependent, ferricyanide and DCIP reductase activities ⩾ 6-fold while stimulating the cyt *c* and INT reductases by approximately 20% (Fig. 3 *B*). Reduction of acceptor in all cases was linear with time over the 2 minutes assay both in the presence (Fig. 3 *B*) and absence (Fig. 3 *A*) of detergent. Stimulation of ferricyanide reductase was independent of the detergent used although Triton X-100 and NP-14 stimulated the activity to a greater extent than did digitonin and CHAPS (Fig. 4). Compared to the rate of reduction in the presence of enzyme, the rate of nonenzymatic ferricyanide reduction by NADH was insignificantly stimulated by detergents. The detergent concentration for Triton X-100 and CHAPS which showed maximal stimulation is 10-fold below the concentration needed to solubilize the activity (data not shown). Thus, the stimulation of activity by detergents suggests the presence of cryptic sites of ferricyanide reduction.

Kinetic analysis of NAD(P)H-dependent, ferricyanide reductase. Reduction of ferricyanide was dependent on protein concentration both in the presence or absence of detergent as illustrated for Triton X-100 (Fig. 5 *A*). The dependence of ferricyanide reductase activity on pyridine nucleotide concentration was investigated. Saturation kinetics were observed for NADH in the absence and for NADH and NADPH in the presence of 0.025% (v/v) Triton X-100 (Figs. 5 *B* and 5 *C*, re-

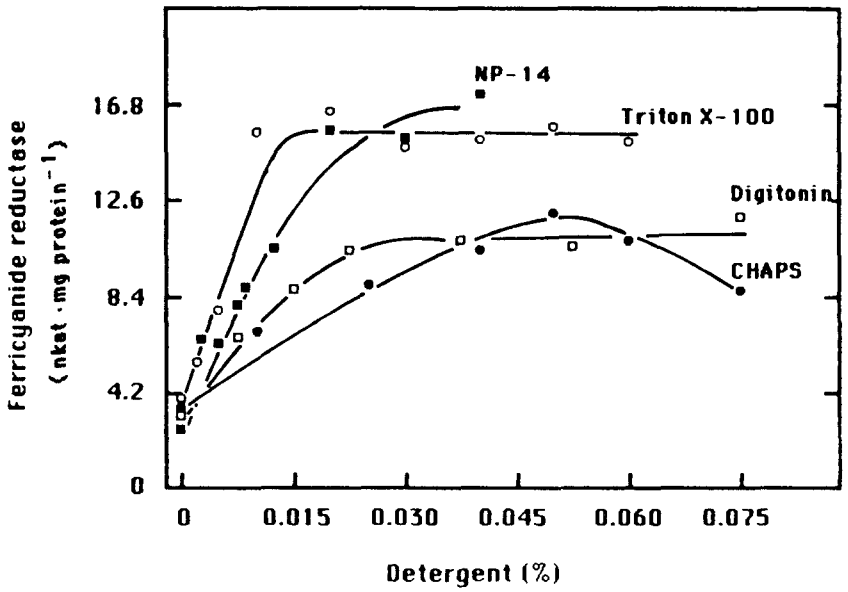

Fig. 4. Analysis of detergents on the NADH-dependent, ferricyanide reductase activity. Reductase activity was assayed in PM as described in the Materials and Methods. The detergents tested were NP-14 (■), Triton X-100 (○), digitonin (□) and CHAPS (●). Detergent concentration for NP-14 and Triton X-100 are percent by volume and for digitonin and CHAPS are percent by weight. Activity in all experiments was conducted using 26 μg membrane protein. Each data point is the average of 3 determinations

spectively). Activity for NADPH-dependent ferricyanide reductase was low and variable between membrane preparations and was therefore not further investigated. Replotting the substrate saturation curves shown in Figs. 5 *B* and 5 *C* using the method of Hanes-Woolf (SEGEL 1975) resulted in linear plots ($r^2 \geqslant 0.99$) for ferricyanide reduction when assayed in the presence of Triton X-100 (Fig. 5 *D*). The Michaelis-Menten constants were $K_m = 20.4\,\mu M$ and $V_{max} = 6.67$ nkat · mg protein^{-1} for NADPH in the presence of Triton, and $K_m = 0.5\,\mu M$ and $V_{max} = 0.83$ nkat · mg protein^{-1} for NADH in the absence and $K_m = 24.3\,\mu M$ and $V_{max} = 20.67$ nkat · mg protein^{-1} for NADH in the presence of Triton.

Analysis of ferricyanide reductase activity in KCl-washed membranes. Both the stimulation of ferricyanide reductase by detergents and the nearly 20-fold increase in K_m for NADH following Triton X-100 treatment suggest the presence of multiple dehydrogenase activities. In an attempt to resolve these activities, membranes were washed by resuspension and pelleting in buffer B containing either low (1 mM) or high (0.5 M) KCl. Membranes washed in high salt showed a decrease in the NADH-dependent activity by approximately one-half and an elimination of the NADPH-dependent activity when assayed in the absence of detergent (Table 6). Washing membranes in low salt was, however, equally as effective in removing NADPH-dependent activity suggesting that this activity was loosely associated with the membrane. Although low salt washing also removed approximately 10% of the NADH-dependent ferricyanide reductase, the activity assayed in the absence of detergent was not affected by the treatment (Table 6). Thus, the NADH-dependent activity removed by low salt washing was likely sequestered within the vesicle. These results show that approximately one-half of the NADH-dependent activity assayed in the absence of detergent and a significant portion of the detergent stimulated NAD(P)H-dependent activity was not salt extractable and thus integral to the membrane.

4. Discussion

With few exceptions (*i.e.* DE LUCA *et al.* 1984, BARR *et al.* 1985 b), NAD(P)H-dependent reductase activity has not been studied in a well characterized PM fraction. The membrane fraction used in this study was shown to be > 90% PM, and based on the latency of the ATPase, the membrane vesicles were shown to be oriented > 90% with the cytoplasmic surface inside. Ferricyanide reduction was greatly stimulated when the activity was assayed in the presence of detergents. We assume that in the absence of detergent, the sites of oxidation and reduction are located on the outer surface of the vesicles since both NADH and fer-

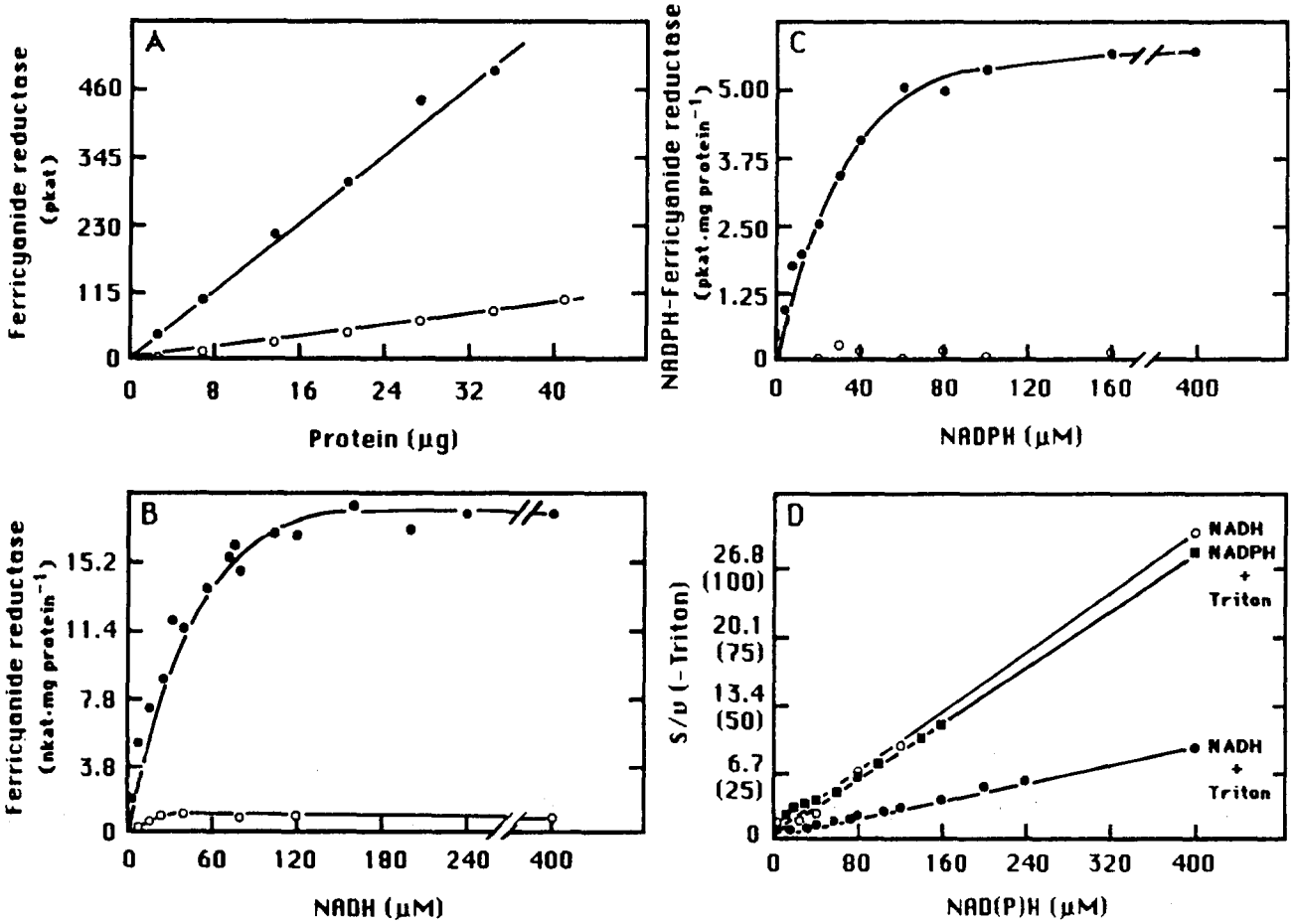

Fig. 5. Analysis of the kinetic properties of an NAD(P)H-dependent, ferricyanide reductase. *A* Analysis of the relationship of membrane protein to NADH-dependent, ferricyanide reductase activity in the presence (●) and absence (○) of 0.025% Triton X-100. *B* Analysis of the dependence of NADH-dependent, ferricyanide reductase activity on the concentration of NADH in the presence (●) and absence (○) of Triton X-100. *C* A similar analysis to that presented in B above but with NADPH substituted for NADH. *D* A replotting of the data presented in B and C using the method of Hanes-Woolf. The numbers in parentheses on the ordinate correspond to NADH-dependent activity measured in the absence of Triton X-100. Kinetic parameters calculated from these plots are: for NADPH-dependent activity plus Triton X-100 (□)$K_m = 20$ and $V_{max} = 6.67$, for NADH-dependent activity plus Triton (●)$K_m = 24$ and $V_{max} = 20.67$, and for NADH-dependent activity minus Triton (○)$K_m = 0.5\,\mu M$ and $V_{max} = 0.83$ nkat · mg protein^{-1}. For the purpose of clarity, several data points at low pyridine nucleotide concentrations were not plotted but were included in K_m and V_{max} calculations

ricyanide are membrane impermeable. External sites for NADH oxidation have been shown in isolated protoplasts (LIN 1982, THOM and MARETZKI 1985) and roots (RUBINSTEIN *et al.* 1984) and for ferricyanide reduction in protoplasts (LIN 1982, THOM and MARETZKI 1985), roots (FEDERICO and GIARTOSIO 1983, RUBINSTEIN *et al.* 1984, QUI *et al.* 1985) and cultured cells (BARR *et al.* 1985 a). Biochemical evidence supporting extracytoplasmic oxidation of NADH by PM has also been presented by DE LUCA *et al.* (1984) and BARR *et al.* (1985 b) and most recently by PUPILLO *et al.* (1986). The detergent stimulation of ferricyanide reductase activity indicates that additional cryptic sites of NAD(P)H oxidation and/or acceptor reduction exist possibly on the cytoplasmic surface of the PM. This finding is in agreement with the published work of DE LUCA *et al.* (1984) who reported a Triton X-100-stimulated, duroquinone-dependent NADH oxidase in a PM fraction from *Cucurbita* and of GREBING *et al.* (1984) who reported a Triton X-100-stimulated, NADH-dependent ferricyanide reductase in erythrocyte membranes.

Kinetic analysis of the pyridine nucleotide saturation plots conform to Michaelis-Menten kinetics. There was no indication of the more complex non-Michaelis-Menten kinetics reported by PUPILLO *et al.* (1986) in zucchini microsomes. The calculated K_m values for NADPH and NADH were approximately 20 µM in the presence of detergent and > 1 µm for NADH in the absence of detergent. K_m values in this concentration

Table 6. *NAD(P)H-dependent, ferricyanide reductase activity in salt-washed membranes. PM were washed in buffer B with or without 0.5 M KCl. Washed membranes were pelleted and the resulting supernatants and pellets were analysed for ferricyanide reductase activity in the presence and absence of 0.025% (v/v) Triton X-100. The results are an average of 3 determinations*

Treatment	Ferricyanide reductase (nkat)			
	NADH-dependent		NADPH-dependent	
	Triton X-100		Triton X-100	
	—	+	—	+
Unwashed	0.77	5.00	0.43	3.73
1 mM KCl				
pellet	0.80	4.43	0.00	2.79
supernatant	—	0.32	—	0.55
0.5 M KCl				
pellet	0.40	3.40	0.03	1.60
supernatant	—	0.75	—	0.73

range are consistent with a physiological role for the PM NAD(P)H oxidoreductase and within the range of values reported in the literature (GREBING *et al.* 1984, PUPILLO *et al.* 1986). The increase in K_m by greater than 20-fold for NADH and a 6-fold increase in V_{max} for the ferricyanide reductase following the addition of Triton X-100 suggests that detergent treatment exposes an enzyme activity distinct from that present in nontreated membranes.

Washing PM with 0.5 M KCl resulted in a 50% reduction in NADH-dependent ferricyanide reductase and elimination of the NADPH-dependent activity assayed in the absence of detergent. In fact, washing membranes in buffer in the absence of KCl also released significant NADH-dependent activity and eliminated NADPH-dependent activity. The portion of the activity removed during washing is thought to be loosely bound to, or sequestered within the membrane and may have originated as cytoplasmic contamination during homogenization. However, nearly one-half of the NADH-dependent reductase assayed in the absence of detergent remains associated with the membrane fraction following salt washing and represents activity tightly bound to the extracytoplasmic surface of the PM.

While NADH-dependent ferricyanide reduction was detected in the presence and absence of detergent following washing, little or no NADPH-dependent ferricyanide reduction was observed in the absence of detergent. Although NADPH was oxidized by intact maize roots (RUBINSTEIN *et al.* 1984), our data indicate that the primary site of NADPH oxidation and/or ferricyanide reduction is not exposed to the outside surface of the PM. This conclusion is supported by the results of QIU *et al.* (1983) and SIJMONS *et al.* (1984) who demonstrated a flow of reducing equivalents from cytoplasmic NADPH to extracytoplasmic ferricyanide in maize roots and from NADPH to ferric iron in iron-deficient bean roots. We speculate that the sites of NAD(P)H oxidation exposed by detergent are on the cytoplasmic surface of the PM may be associated with transmembrane proteins involved in transport of reducing equivalents to the apoplast.

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